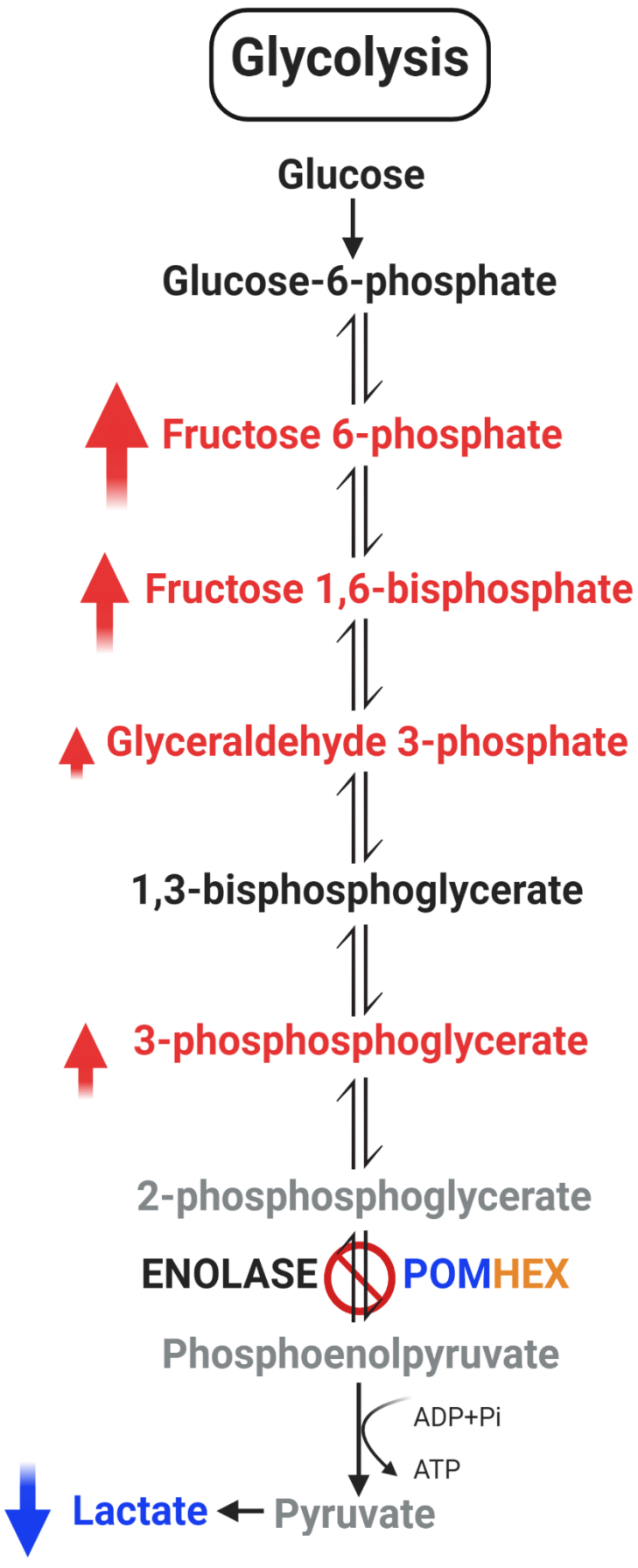
Supplementary figures

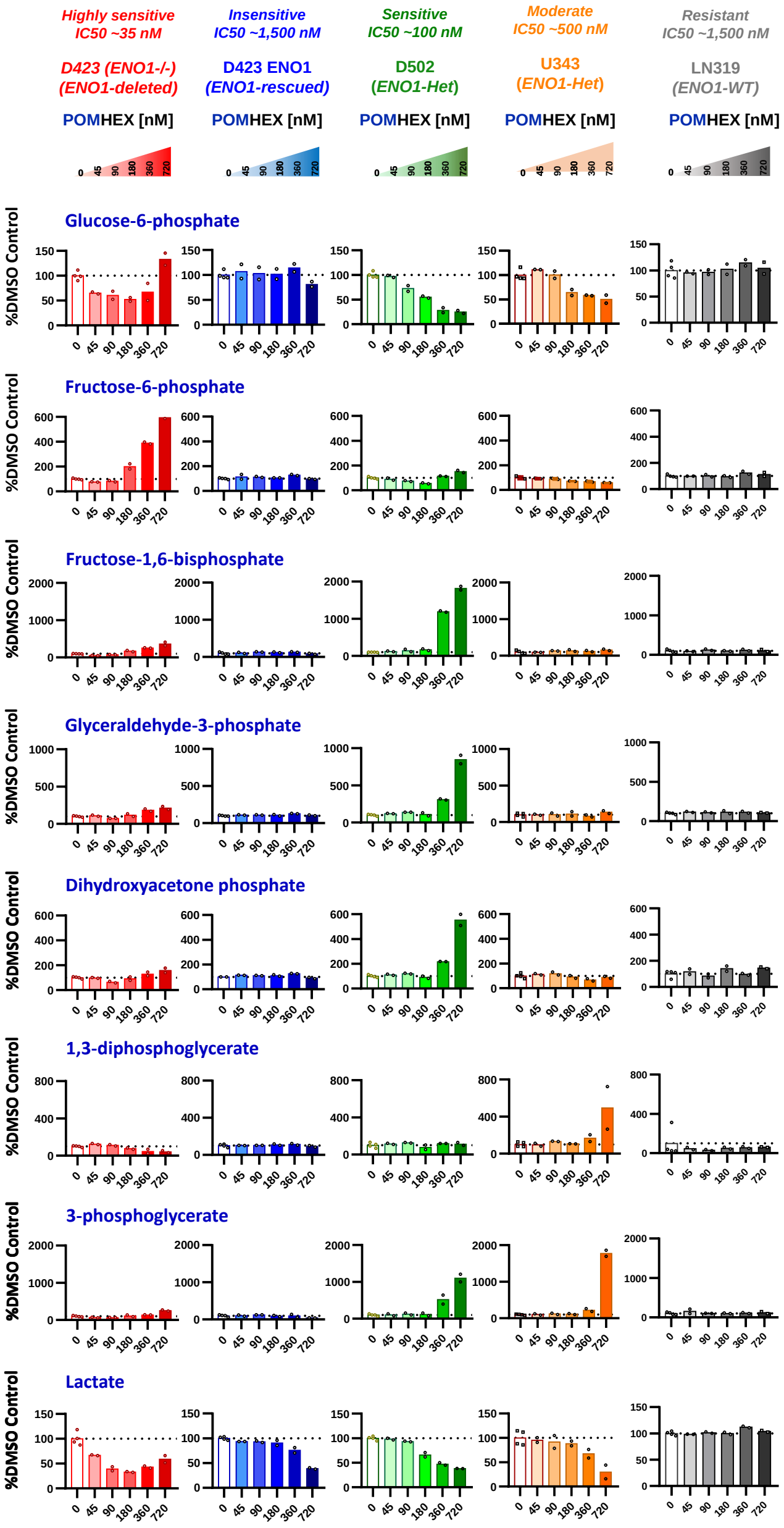
Supplemental Figure S1. POMHEX treatment results in accumulation of glycolytic metabolites upstream of enolase
Supplemental Figure S2. POMHEX treatment leads to an overall reduction in TCA cycle metabolites.
Supplemental Figure S3. Rescue of POMHEX toxicity by different anaplerotic substrates.
Supplemental Figure S4. Pyruvate rescues POMHEX toxicity in glioma cells with homozygous and heterozygous deletion of ENO1
Supplemental Figure S5. Glioma cells exhibit glutamine auxotrophy in vitro
Supplemental Figure S6. CB-839 toxicity is exaggerated under pyruvate free conditions and reversed by addition of anaplerotic substrates
Supplemental Figure S7. Metabolomic analysis of HEX treated ENO1 deleted subcutaneous tumors (Metabolon-Inc) platform.

Figure S1

A


Glycolysis
Glucose
Glucose-6-phosphate
Fructose 6-phosphate
Fructose 1,6-bisphosphate
Glyceraldehyde 3-phosphate
1,3-bisphosphoglycerate
3-phosphosphoglycerate
2-phosphosphoglycerate
ENOLASE
POMHEX
Phosphoenolpyruvate
ADP+Pi
ATP
Lactate
Pyruvate

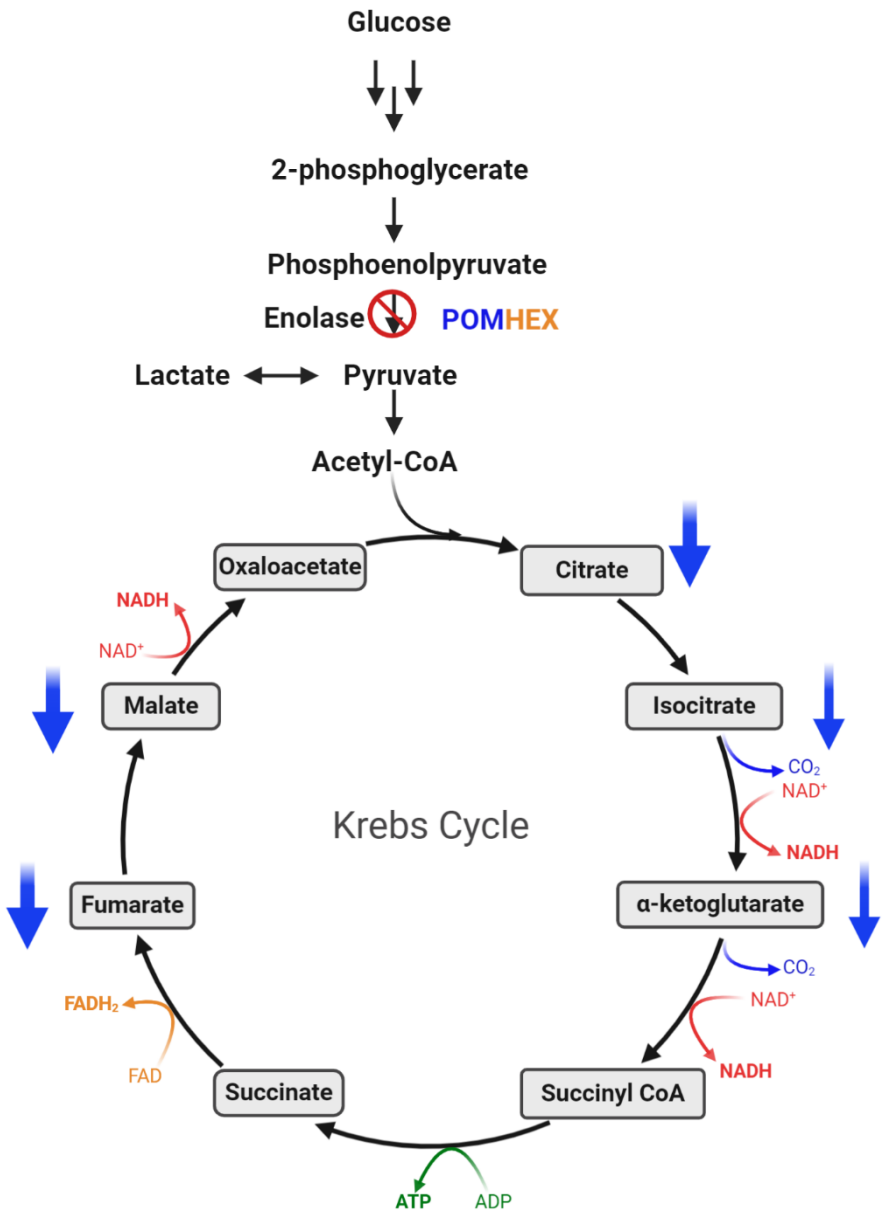
B


Highly sensitive
IC50 ~35 nM
D423 (ENO1-/-)
(ENO1-deleted)
Insensitive
IC50 ~1,500 nM
D423 ENO1
(ENO1-rescued)
Sensitive
IC50 ~100 nM
D502
(ENO1-Het)
Moderate
IC50 ~500 nM
U343
(ENO1-Het)
Resistant
IC50 ~1,500 nM
LN319
(ENO1-WT)
POMHEX [nM]
0 45 90 180 360 720
%DMSO Control
Glucose-6-phosphate
Fructose-6-phosphate
Fructose-1,6-bisphosphate
Glyceraldehyde-3-phosphate
Dihydroxyacetone phosphate
1,3-diphosphoglycerate
3-phosphoglycerate
Lactate

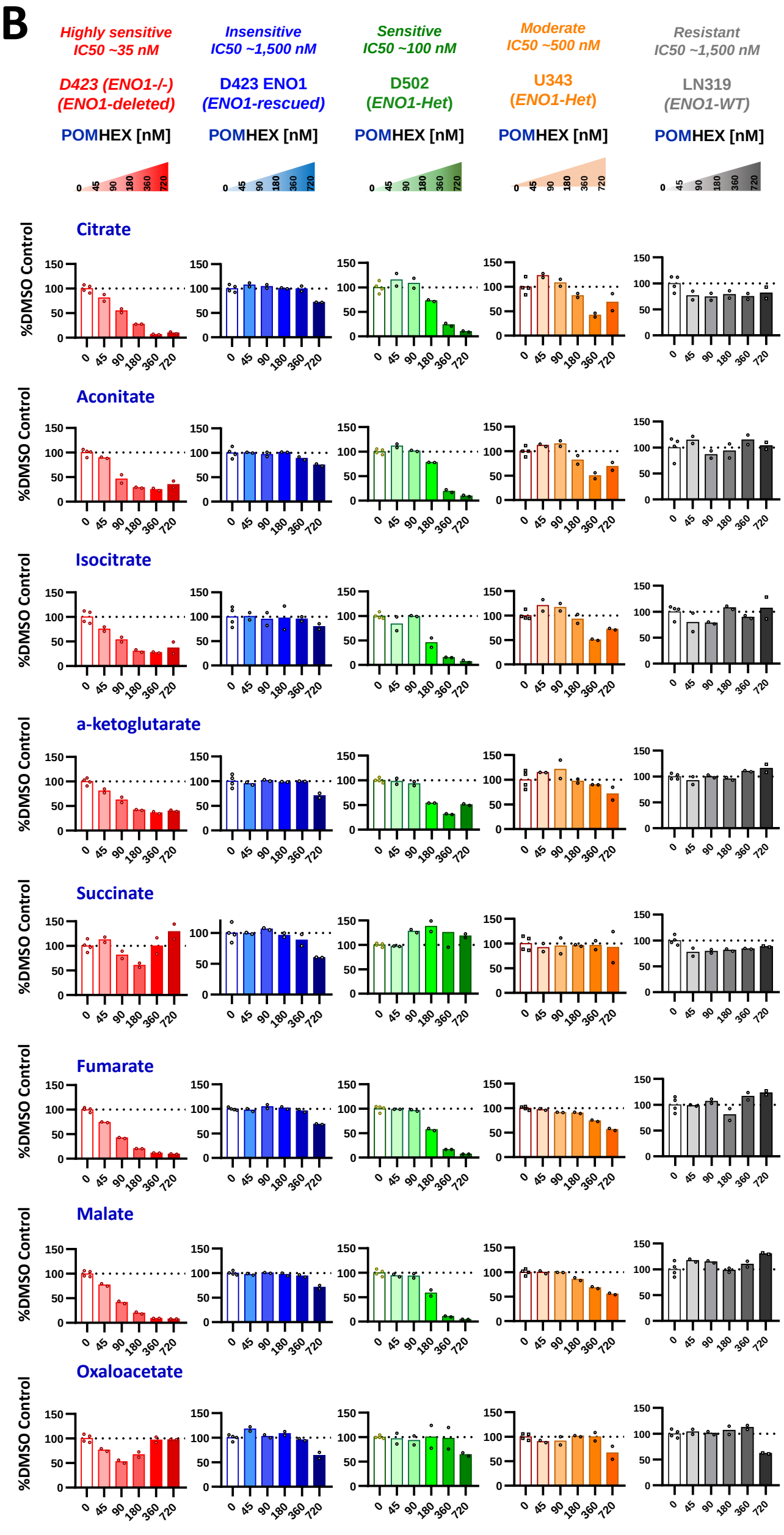
Supplemental Figure S1.**POMHEX treatment elevates the levels of glycolytic intermediates upstream of enolase**. **A.** Schematic showing the glycolytic intermediates that are altered by the POMHEX treatment. **B.** *ENO1* homozygously deleted (D423,red), *ENO1*-isogenic rescue (D423 *ENO1*, blue), *ENO1* heterozygous (D502, green; U423, orange) and *ENO1* wild type (LN319, grey) were treated with serial dilution of enolase inhibitor POMHEX for 72 hours and metabolomics analysis was performed on the extracted intracellular metabolites (N=2 biological replicates). The level of each metabolite is expressed relative to the DMSO control group. Treatment with POMHEX leads to an overall and significant accumulation of metabolites upstream of enolase reaction. Note that the degree of metabolite accumulation correlates with the sensitivity of each cell line to POMHEX (**Figure 2**).

Figure S2

A


Glucose
2-phosphoglycerate
Phosphoenolpyruvate
Enolase
POMHEX
Lactate
Pyruvate
Acetyl-CoA
Oxaloacetate
Citrate
NADH
NAD+
Malate
Isocitrate
CO2
NAD+
NADH
Krebs Cycle
Fumarate
α-ketoglutarate
CO2
NAD+
FADH2
FAD
NADH
Succinate
Succinyl CoA
ATP
ADP

B


Highly sensitive
IC50 ~35 nM
D423 (ENO1-/-)
(ENO1-deleted)
Insensitive
IC50 ~1,500 nM
D423 ENO1
(ENO1-rescued)
Sensitive
IC50 ~100 nM
D502
(ENO1-Het)
Moderate
IC50 ~500 nM
U343
(ENO1-Het)
Resistant
IC50 ~1,500 nM
LN319
(ENO1-WT)
POMHEX [nM]
0 45 90 180 360 720
Citrate
Aconitate
Isocitrate
a-ketoglutarate
Succinate
Fumarate
Malate
Oxaloacetate
%DMSO Control
150
100
50
0

Supplemental Figure S2. **TCA cycle metabolites decrease in response to enolase inhibitor POMHEX**. **A.** Schematic showing the entry of pyruvate derived carbons as acetyl coenzyme A and glutamate carbons as a-ketoglutarate into the TCA cycle. **B.** *ENO1* homozygously deleted (D423,red), *ENO1*-isogenic rescue (D423 *ENO1*, blue), *ENO1* heterozygous (D502, green; U343, orange) and *ENO1* wild type (LN319, grey) were treated with the enolase inhibitor POMHEX at the indicated doses for 72 hours and polar metabolites were extracted (N=2 biological replicates). Metabolites are expressed relative to the DMSO control. Metabolomics analysis show an overall decrease in the levels of TCA cycle metabolites downstream of the enolase reactions. The degree of disruption in the levels of TCA cycle intermediates correlates with the sensitivity of each cell line to the inhibitor (**Figure 2**).